Patent Application
Docket No. SPO-121

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Examiner	:	Satyendra K. Singh
Art Unit	:	1657
Applicant	:	Hisae Kume *et al.*
Serial No.	:	10/535,585
Conf. No.	:	7558
Filed	:	May 18, 2005
For	:	Nutritional Compositions

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

EXPERT DECLARATION OF MS. HISAE KUME UNDER 37 CFR §1.132

Sir:

I, Hisae Kume, hereby declare:

THAT, I am a co-inventor of the above-referenced patent application;

THAT, I have reviewed the Office Action mailed November 26, 2007, along with the references cited therein;

THAT, I have extensive experience in the field of nutritional compositions;

And being thus duly qualified, do further declare as follows:

1. The attached experimental results (see attached Appendix A) show that fermented milk products contain a substantial amount of undegraded proteins. Test 1 reveals gel filtration chromatography comparing unfermented milk with fermented milk. HPLC patterns for each sample are shown in Figure 1. The prominent chromatography peaks represent the major protein constituents of milk: casein, β-lactoglobulin, and α-lactalbumin. Electrophoretic analysis as shown in Figure 2 corroborates these results. Protein bands corresponding to casein, β-lactoglobulin, and α-lactalbumin remain in both skimmed milk and fermented milk. The results show the retention of between 88-95% of whole protein in fermented milk following the fermentation process.

2. Whey protein hydrolysates display markedly different protein content characteristics compared to the fermented milk described above. Figure 2 includes analysis of a whey protein hydrolysate. The results confirm significant whole protein degradation, as evidenced by the lack of any visible protein banding for the whey protein hydrolysate as compared to the fermented milk. Test 3 substantiates this finding using gel filtration chromatography. Figure 3 illustrates the extent of whole protein degradation of whey protein hydrolysate. The chromatographic peak noted at 1 kDa verifies the degradation and lack of whole proteins of milk, such as casein, β- lactoglobulin, and α-lactalbumin.

I hereby further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

By: _____ Date: August 8, 2008

APPENDIX

1. Comparison between proteins in fermented milk and skimmed milk powder
2. Comparison between fermented milk and whey protein hydrolysate

Test 1: Analysis 1 using gel filtration chromatography

In order to compare the protein constitution of fermented milk and skimmed milk powder, analysis using gel filtration chromatography was performed.

Conditions for analysis

Column: TOSOH TSK-GEL super SW2000 (ϕ4.6 x 300 mm)

Mobile phase: 8M urea/0.2M phosphate buffer (pH6.7) = 1/2, 0.35 ml/min

Detection wavelength: UV 280 nm

Sample: (1) skimmed milk before fermentation, (2) fermented milk 1-3, 10 mg /ml each

Results

HPLC patterns for each sample are shown in Fig 1.

In this experiment, the retention time was divided into four (Retention Times 1-4), and their area ratio (%) was calculated.

	Total of Retention Time 1 and Retention Time 2	Retention Time 3	Retention Time 4
Skimmed milk before fermentation	83.8	7.2	9.0
Fermented milk 1	73.8 (88% of that before fermentation)	12.2	14.0
Fermented milk 2	77.2 (92% of that before fermentation)	13.3	9.6
Fermented milk 3	79.6 (95% of that before fermentation)	10.7	9.8
Estimated molecular weight (Da)*	> 30000 (Retention Time 1) 30000-10000 (Retention Time 2)	10000-1000	<1000

*Estimated from the application data of the analytical column

Test 2: Analysis by electrophoresis

Skimmed milk before fermentation, fermented milk 3, and trypsin hydrolysate of whey protein were electrophoresed to compare the proteins contained in each sample.

Conditions for analysis

Gel plate: Ready Gel 10% Tris-HCl Gel (Bio-Rad)

Electrophoresis buffer: Tris/Glycine/SDS

Electrophoresis condition: 30 mA, 90 min

Staining method: 1-hour staining with Coomassie Brilliant Blue, overnight decolorization

Results

The electrophoresis patterns for each sample are shown in Fig 2. As in AS Egito *et al.*, J Dairy Sci, 85, pp. 697-706 (2002), for skimmed milk before fermentation and fermented milk 3, bands near 30 kDa, which are mainly ascribable to Casein, were observed. Furthermore, other bands near 15 kDa, which correspond to β-lactoglobulin and α-lactalbumin, were observed (HM Farrell, Jr *et al.*, J Dairy Sci, 87, pp. 1641-1674 (2004)). On the other hand, no bands were observed for trypsin hydrolysate of whey protein. In addition, bands corresponding to aggregated protein of several hundred kDa or more were not observed in either of the samples.

Test 3: Analysis 2 using gel filtration chromatography

Analysis using gel filtration chromatography was performed to determine the molecular weight distribution of the proteins in trypsin hydrolysate of whey protein.

Conditions for analysis

Column: TOSOH TSK-GEL SWXL (ϕ 7.5 x 30 mm)

Mobile phase: 45% acetonitrile, 0.1% trifluoroacetic acid, 0.5 ml/min

Detection wavelength: UV 215 nm

Sample: Trypsin hydrolysate of whey protein (4-hour treatment with trypsin at 58°C)

Results

The result is shown in Fig 3. The average molecular weight of the tested sample was 990 Da.

Discussion 1: Comparison between proteins in fermented milk and skimmed milk

The main proteins contained in cow milk are as follows:

Casein: About 80%, molecular weight of each molecular species of α, β, γ, κ ranges from about 19 kDa to 25 kDa (theoretical value calculated from the genetic sequence)

β-lactoglobulin: About 10%, molecular weight of about 18 kDa (theoretical value calculated from the genetic sequence)

α-lactalbumin: About 4%, molecular weight of about 14 kDa (theoretical value calculated from the genetic sequence)

The peak that appears in Retention Time 2 of Test 1 is believed to be ascribable to these proteins.

When the sample subjected to analysis is dense, protein aggregates may form. In that case, the aggregated protein elutes at a shorter retention time than that of the actual molecular weight. In Test 1, the peak that appears in Retention Time 1 is believed to be ascribable to aggregated proteins. In electrophoresis of Test 2, the effect of protein aggregation is low, and therefore, proteins that mainly correspond to Retention Time 2 were detected both in skimmed milk before fermentation and fermented milk.

Accordingly, in order to evaluate the amount of "whole protein" contained in the samples, those eluted at Retention Times 1 and 2 were subjected to evaluation. Three types of fermented milk were prepared and compared with skimmed milk before fermentation, and it was revealed that 88-95% of the proteins remain in fermented milk 1-3.

From these results, it was proven that fermented milk contains substantial amount of proteins contained in skimmed milk before fermentation.

Discussion 2: Comparison between fermented milk and whey protein hydrolysate

In Test 2, equal amounts of fermented milk and trypsin hydrolysate of whey protein were compared. Substantial amount of proteins was detected from fermented milk; however, bands stained by Coomassie Blue (which stains proteins) were not detected for trypsin hydrolysate of whey protein.

Furthermore, in Test 3, the average molecular weight of trypsin hydrolysate of whey protein was about 990 Da, which is greatly different from that of fermented milk.

These results prove that fermented milk proteins and whey protein hydrolysates differ in composition.

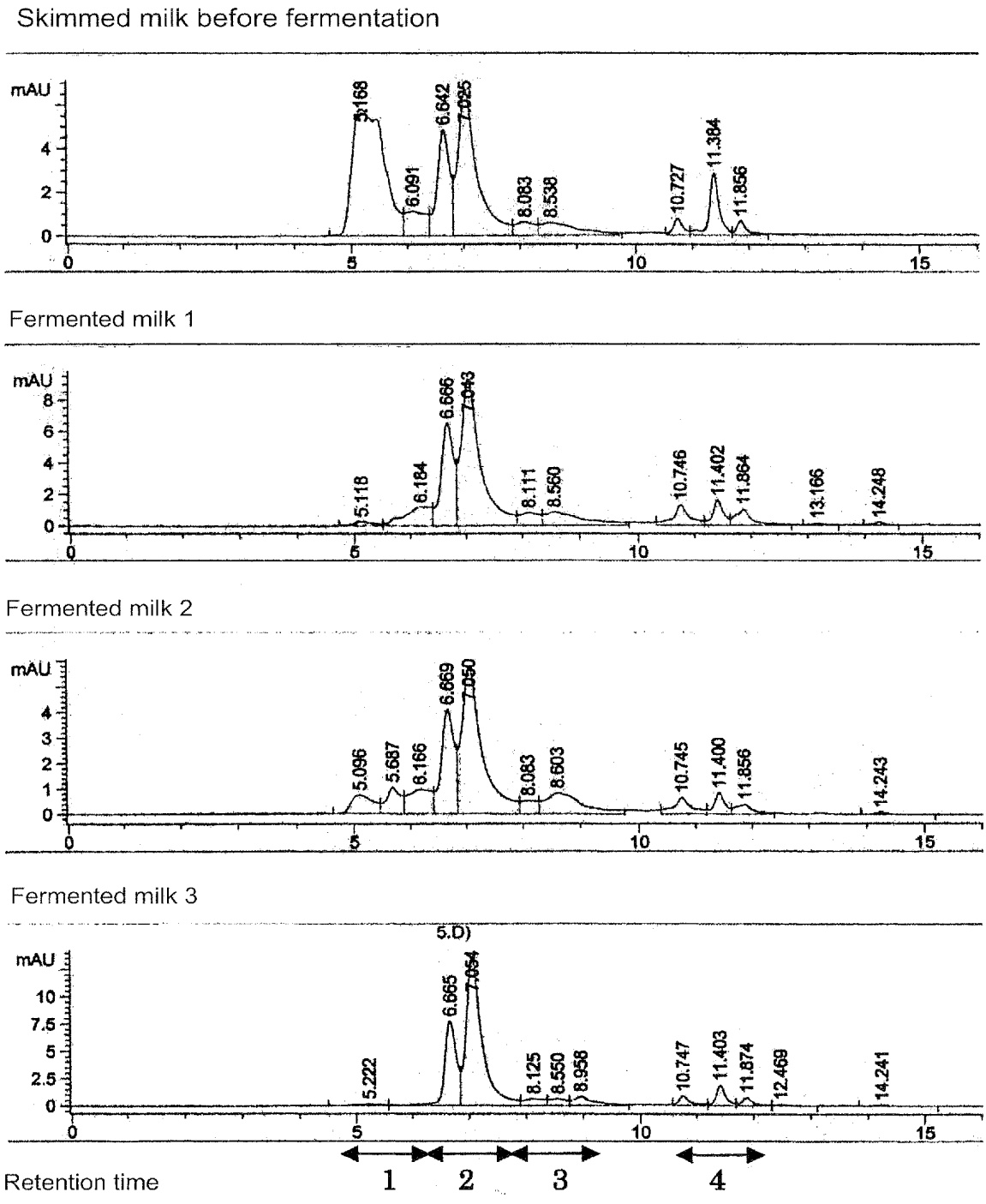
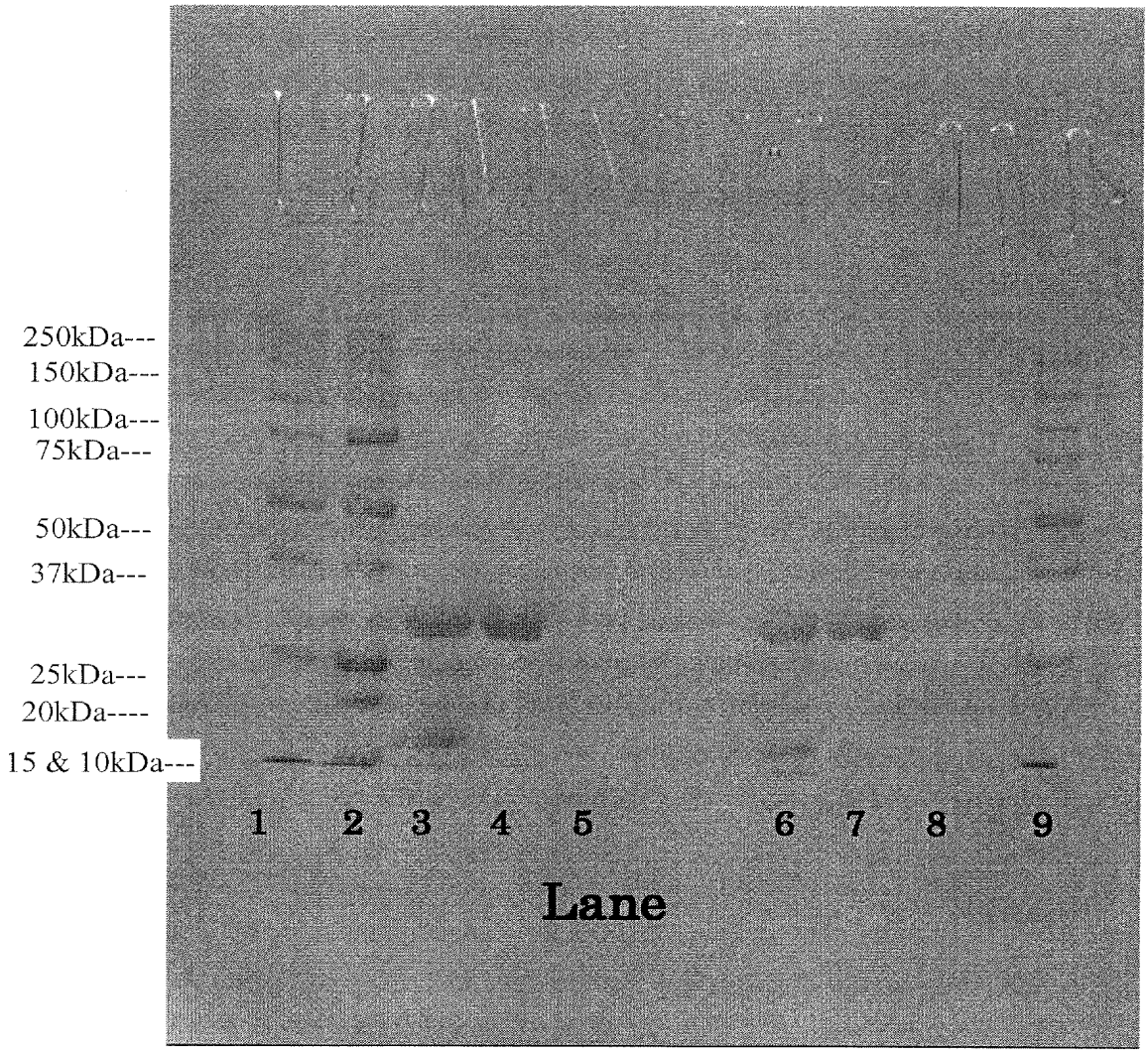


Fig 1. Analysis 1 using gel filtration chromatography


250kDa---
150kDa---
100kDa---
75kDa---
50kDa---
37kDa---
25kDa---
20kDa----
15 & 10kDa---
1 2 3 4 5 6 7 8 9
Lane

Lane	Sample	
1	Marker 1	
2	Marker 2	
3	Skimmed milk before fermentation	10 μg/Lane
4	Fermented milk 3	10 μg/Lane
5	Trypsin hydrolysate of whey protein	10 μg/Lane
6	Skimmed milk before fermentation	5 μg/Lane
7	Fermented milk 3	5 μg/Lane
8	Trypsin hydrolysate of whey protein	5 μg/Lane
9	Marker 1	

Fig 2. Analysis by electrophoresis

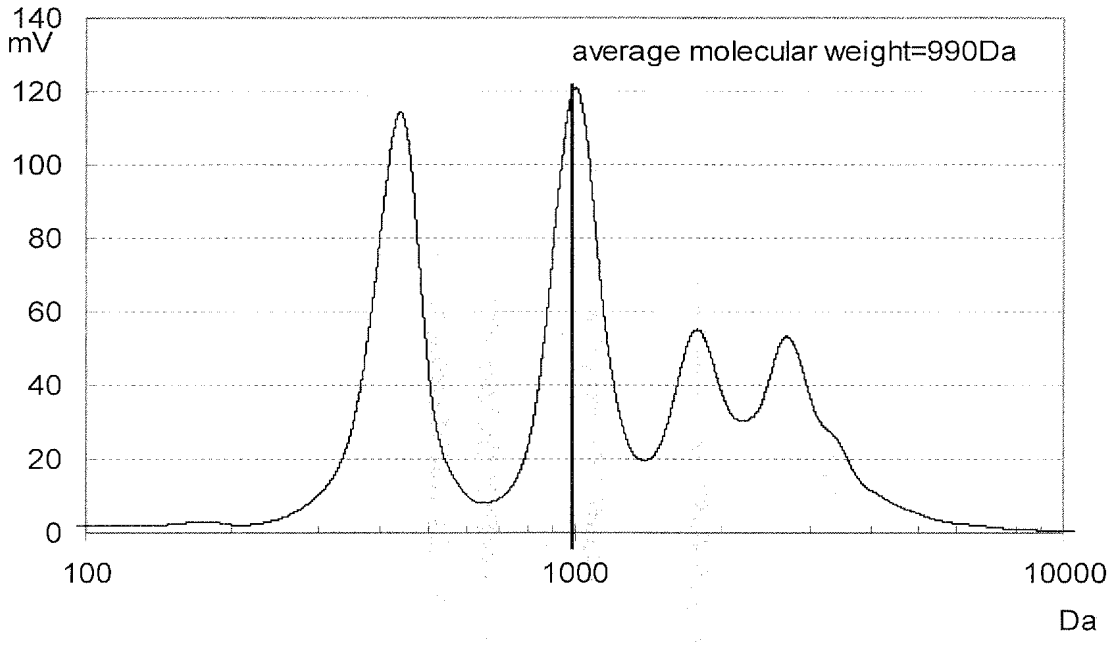


Fig 3. Analysis 2 using gel filtration chromatography